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Domain Transcription Factor Associated with Breast
Cancer: Effects of ESX/DNA Complex Disruption

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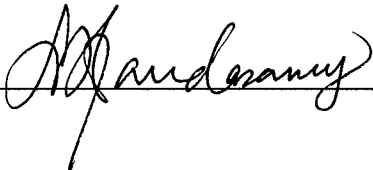
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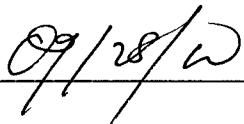
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13. ABSTRACT (Maximum 200 Words) DNA binding agents are being evaluated for their ability to disrupt transcription factor binding to DNA and down regulate cancer associated gene expression. Target gene is HER2/neu, which is overexpressed in 30% of breast cancers. Drugs with different modes of binding as well as different sequence preference are being evaluated. Polyamides, a novel group of DNA binding agents with sequence specific binding, are also under evaluation. Cell-free assays such as mobility shift and cell-free transcription are being employed to assess agents' ability to disrupt transcription factor binding. Agents showing potential in cell-free assays were evaluated in whole cell assays using northern analysis. Results in mobility shift assays indicate that sequence specific binding agents inhibit transcription factor DNA complexes better the sequence preference agents by an order of magnitude. In cell-free transcription assays there is less difference between the two types of drugs. Some sequence preference agents are very effective at inhibiting gene expression in whole cells, while first generation polyamides show limited ability to diminish gene expression.				
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FOREWORD

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Stephanie Leslie 9/1/99
PI - Signature Date



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Annual Summary

Introduction:

Regulation of expression of cancer associated genes by anti-cancer agents is our current area of research. Gene expression requires the association of transcription factors both general and gene specific to the promoter region to allow for transcription of that gene. Disruption/prevention of transcription factor binding to a gene promoter should decrease gene expression. Our current studies, evaluation of DNA binding agents with different modes of binding (reversible/intercalating) and different sequence preference (GC vs. AT vs. sequence specific) for their ability to target and potentially down regulate gene expression. The target is HER2/neu gene expression since it is found overexpressed in approximately 30% of all breast cancer cases. Recently an ETS family transcription factor, ESX, has been identified as a possible regulatory transcription factor of the HER2/neu gene. Our goal is to assess DNA binding drugs for their ability to interfere with transcription factor (ESX) binding to the target gene's promoter region (HER2/neu). This work has begun to uncover drug DNA binding motifs that can be exploited to develop new drugs or modify existing agents that could target HER2/neu promoter expression. Also a new novel group of DNA binding agents, polyamides which are highly sequence specific, are also being assessed to determine if these agents might be more specific and potent inhibitors of target gene expression.

Annual Summary

Body:

DNA binding agents possess different DNA binding characteristics. Mode of binding is one defining characteristic of drugs. A few examples include binding to the minor groove of DNA or intercalation between the base pairs of DNA. Another defining characteristic is preference of binding to DNA, either GC rich or AT rich. In addition, early during this grant, an opportunity for collaboration with a scientist who has developed a new group of novel DNA binding agents presented itself. Dr. Peter Dervan from the Division of Chemistry and Chemical Engineering at the California Institute of Technology has developed a group of agents called polyamides. Polyamides contain N-methylimidazole (Im) and N-methylpyrrole (Py) amino acids combined in antiparallel side-by-side combination, which fit into the minor groove of DNA. Combinations of these amino acids possess the ability to chemically "read" and bind to specific DNA sequences. For example, Im/Py recognizes GC base pairs, Py/Im recognizes CG base pairs while a Py/Py is degenerate for AT and TA base pairs. During this collaboration Dr. Dervan's group has designed agents targeted to the ESX binding site of the HER2/neu promoter. Assessment of sequence preference and sequence specific agents may elucidate drug characteristics that allow for more specific or potent inhibition of cancer associated gene expression.

AIM 1: Identification of DNA binding agents that interfere with the binding of ESX to HER/2neu promoter DNA in cell-free assays:

- In accordance with Task 1 of the Statement of Work, months 1-6, I have been assessing DNA binding agents to uncover DNA binding to develop new drugs or modify existing agents that could target HER2/neu promoter expression. An assay for quantitative analysis of drug inhibition of transcription factor/DNA complex formation is the mobility shift assay (MSA). Using purified components, ESX protein and an oligonucleotide containing the ESX binding site, improves the likelihood that differences seen between the DNA binding agents is due to that agent's ability to disrupt ESX binding to DNA and not interactions with other cellular components. Bacterially expressed purified ESX protein is combined with radiolabelled oligonucleotide containing its consensus DNA binding site from the HER2/neu promoter region. The reaction is loaded onto a native polyacrylamide gel and electrophoresed to separate complexed (near the top of gel) from the unbound DNA (near the bottom of gel). Relative amount of shifted vs. unshifted DNA is determined by densitometric scans of autoradiograms. DNA binding agents can be employed in this assay by incubation with the DNA prior to (for prevention of complex formation) or subsequent to (for disruption of pre-formed complex) protein addition. A series of drug concentration is evaluated and quantitatively analyzed. From this data IC₅₀ values (the molar concentration of drug required to inhibit complex formation by 50%) can be obtained to identify and compare compounds. Agents evaluated thus far are presented in Table 1 along with IC₅₀ values for prevention

of complex formation. Assessment of agents for their ability to disrupt pre-formed transcription factor/DNA complexes is underway.

Table 1: The IC₅₀ values for DNA binding agents' ability to prevent transcription factor/DNA complex formation.

AGENT	BINDING MODE	SEQUENCE PREFERENCE	IC ₅₀
NOGALAYMCIN	INTERCALATING	GC RICH	ND
LUCANTHONE	INTERCALATING	AT RICH	ND
HEDAMYCIN	INTERCALATING	GC RICH	0.53 UM
ADRIAMYCIN	INTERCALATING	MIXED PREFERENCE	ND
MITOXANTRONE	INTERCALATING	MIXED PREFERENCE	ND
DISTAMYCIN	MINOR GROOVE	AT RICH	0.75 UM
CC-1065	MINOR GROOVE	AT RICH	ND
CHROMOMYCIN*	MINOR GROOVE	GC RICH	ND
HOECHST'S 33342	MINOR GROOVE	AT RICH	0.63 UM
POLYAMIDE 2	MINOR GROOVE	SEQUENCE SPECIFIC	0.010 UM
POLYAMIDE 22	MINOR GROOVE	SEQUENCE SPECIFIC	0.044 UM

* = Optimization of MSA for chromomycin is currently being examined.
 Chromomycin activity is Mg⁺² dependent however Mg⁺² tends to be complex formation inhibitory by itself.
 ND = not done yet

- In accordance with Task 1 of the Statement of Work, months 7-12, DNA footprinting for sequence specific agents, polyamides, are completed at this time. I shall assess sequence preference agents' binding location only for agents that have the desired effect of decreasing target gene expression. These will begin within a year when assessment of all drugs to be studied should be completed.

AIM 2: Effects of DNA binding agents on ESX regulated expression of HER2/neu:

- In accordance with Task 2 of the Statement of Work, months 13-24, cell-free transcription assays of some agents has been accomplished while others are currently on-going for agents with activity in MSA. This cell-free assay employs the use of nuclear lysates to create an environment similar to whole cells. SKBR3 cells that overexpress HER2/neu and express ESX protein are used for these experiments. Briefly, this assay is performed by incubation of DNA template (containing HER2/neu promoter region) and drug prior to addition of radiolabelled nucleotides and nuclear lysates. When the reaction is complete samples are run on a denaturing polyacrylamide gel and the radiolabelled transcript from the DNA template can be visualized by autoradiography. Samples are normalized by an internal control with an end-radiolabelled transcript of a different molecular weight than the HER2/neu transcript. Densitometric scanning of autoradiograms allows for quantitative analysis of DNA binding agents' ability to prevent HER2/neu expression in a cell-free assay and determination of IC₅₀ values (the molar concentration of drug to inhibit complex formation by 50%).

Table 2: The IC50 values for DNA binding agents' ability to prevent transcription in cell-free transcription assay.

AGENT	BINDING MODE	SEQUENCE PREFERENCE	IC50
NOGALAYMCIN	INTERCALATING	GC RICH	ND
LUCANTHONE	INTERCALATING	AT RICH	ND
HEDAMYCIN	INTERCALATING	GC RICH	8.0 UM
ADRIAMYCIN	INTERCALATING	MIXED PREFERENCE	ND
MITOXANTRONE	INTERCALATING	MIXED PREFERENCE	ND
DISTAMYCIN	MINOR GROOVE	AT RICH	5.4 UM
CC-1065	MINOR GROOVE	AT RICH	ND
CHROMOMYCIN	MINOR GROOVE	GC RICH	ND
HOECHST'S 33342	MINOR GROOVE	AT RICH	2.8 UM
POLYAMIDE 2	MINOR GROOVE	SEQUENCE SPECIFIC	4.5 UM
POLYAMIDE 22	MINOR GROOVE	SEQUENCE SPECIFIC	17.2 UM

ND = not done yet

- In accordance with Task 2, months 25-36, at this time the biological reagents are being created for use. When this work is complete DNA binding agents will be assessed in this system. Based on promise in cell-free assays, we have added in direct assays for evaluation of drugs as inhibitors of transcription factor binding in whole cells. This is defined in Task 3.
- Task 3, to assess DNA binding agents' ability to decrease targeted gene expression in whole cell assays. These cellular assays included (I) northern analysis for detection of decreased RNA expression in whole cells and (II) nuclear run-on to test the agents for ability to decrease rate of transcription of gene target.

I: Agents tested thus far which have activity in both mobility shift and cell-free transcription assays were then assessed for their ability to work in a cellular environment by northern analysis. The northern assay involves treatment of whole cells with drug followed by isolation of total RNA. This RNA is run on a denaturing agarose gel and transferred to a nylon membrane. The membrane is then hybridized with an end-radiolabelled cDNA of choice. For these studies cDNA included gene of interest, HER2/neu, and a housekeeping gene, GAPDH. Inclusion of GAPDH probe will help determine if the drug is inhibiting general vs. specific gene transcription. Blots are then quantitatively analyzed using densitometric scanning of autoradiograms.

Complications within this assay were discovered due the mRNA half-life of HER2/neu. The use of SKBR3 cells, known to overexpress the target gene, are incubated with series of concentrations of DNA binding agents for a series of different time points. Probing for HER2/neu mRNA is complicated by the fact that the mRNA half-life is 7.5 hours long. Hence, if short time drug exposure is desired mRNA that was made previous to drug addition would still be present in the total RNA at time of harvesting making it appear that the agent may not have much of an effect. To circumvent this complication longer time of drug exposure was implemented. For example, if drug is added at time zero and assuming that a drug could inhibit gene expression by 100% the earliest time that would allow for

detection of inhibition (without the complication of mRNA previously made) would be 16 hours. That is ~8 hours for previous message to disappear and another 8 hours to see inhibition of message production during drug treatment. Time points for these assays were therefore set for 24, 48 and 72 hours. IC₅₀ values of drugs assessed thus far are included in Table 3.

Table 3: The IC₅₀ values for inhibition of HER2/neu expression in whole cells by Northern analysis.

AGENT	BINDING MODE	SEQUENCE PREFERENCE	IC ₅₀ – HER2/neu	IC ₅₀ - GAPDH
NOGALAYMCIN	INTERCALATING	GC RICH	NO EFFECT	NO EFFECT
LUCANTHONE	INTERCALATING	AT RICH	ND	ND
HEDAMYCIN	INTERCALATING	GC RICH	NO EFFECT	NO EFFECT
ADRIAMYCIN	INTERCALATING	MIXED PREFERENCE	ND	ND
MITOXANTHONE	INTERCALATING	MIXED PREFERENCE	ND	ND
DISTAMYCIN	MINOR GROOVE	AT RICH	38 UM - 72 HRS	167 UM – 72 HRS
CC-1065	MINOR GROOVE	AT RICH	ND	ND
CHROMOMYCIN	MINOR GROOVE	GC RICH	0.035 UM - 24 HRS	0.071 UM – 24 HRS
HOECHST'S 33342	MINOR GROOVE	AT RICH	8.8 UM - 24 HRS	8.6 UM – 24 HRS
POLYAMIDE 2	MINOR GROOVE	SEQUENCE SPECIFIC	NO EFFECT	NO EFFECT
POLYAMIDE 22	MINOR GROOVE	SEQUENCE SPECIFIC	NO EFFECT	NO EFFECT

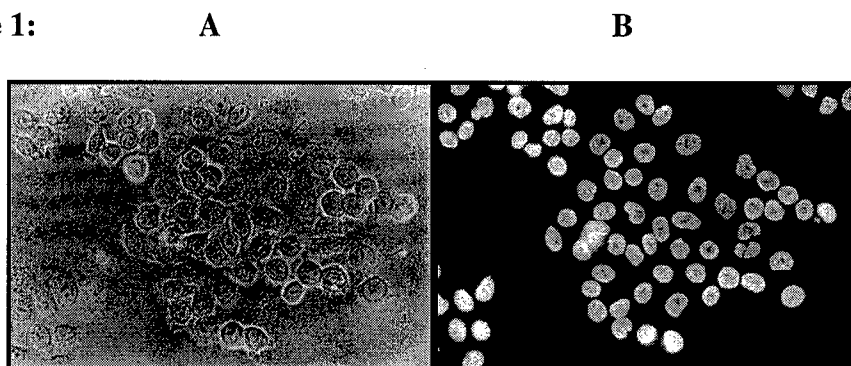
ND = not done yet

Cytotoxicity assays were incorporated to help normalize any drug activity seen in whole cells. Moreover, a lack of cytotoxicity may indicate that a drug cannot effectively enter whole cells. Such is the case for Polyamide 2, though possessing inhibitory activity in mobility shift and cell-free transcription assays did not appear to have an effect on gene expression by northern analysis. Use of Polyamide 2 in cytotoxicity assays showed no detrimental effects on the cells.

Concern for cellular uptake of these new sequence specific DNA binding agents led us to ask our collaborator to create a fluorescently labeled version of Polyamide 2. This new agent was named Polyamide 22. Assessment of Polyamide 22 in cell-free assays was performed due to concern that the fluorescent tag would interfere with the agent's ability to bind to its target sequence. Both mobility shift and cell-free transcription assays showed that Polyamide 22 was four-fold less potent but still capable of functioning similar to Polyamide 2. Since work like this had not been attempted in our laboratory before, I developed a method for determining if Polyamide 22 was being taken into the cells. SKBR3 cells were plated onto coverslips and allowed to grow for several days. Cells are then treated with Polyamide 22 at 0.5 uM for 4 and 24 hours. Coverslips were then harvested, fixed and mounted to slides. Drug uptake was determined by use of a fluorescent microscope available within the department. Figure 1 contains photographs taken from the 4 hour drug treatment. Figure 1A depicts a field of cells using phase and 1B is the exact same field using fluorescence. It can easily be seen from these photographs that the drug, which appears white, is traversing the cytoplasmic membrane as well as the nuclear membrane. However, once the drug has reached the nucleus it seems to clearly avoid the nucleoli. The question still remains as to what, if anything, the drug is doing once it reaches the nucleus of the cell. Further studies, such as electron

microscopy and fluorescent confocal microscopy, are going to be started in the near future to evaluate the Polyamide activity.

Figure 1:



II: In an attempt to circumvent the long half-life of HER2/neu mRNA other, more sensitive assays have been considered for testing of these agents in whole cells. I have been optimizing a nuclear run-on assay that has never been used in our lab. Nuclear run-on (also known as run-off) assay allows for detection of decrease in rate of transcription of a gene of interest at a single moment in time. Transcripts that were initiated prior to cell lysis are elongated in the reaction and incorporate radiolabelled nucleotides. Radiolabelled transcripts are allowed to hybridize to a nitrocellulose membrane containing cDNAs of choice. Densitometric scanning of autoradiograms allow for quantitative analysis of transcriptional inhibition of gene expression. After researching this method, I discovered that further optimization was needed to use SKBR3s since they seemed sensitive to cell lysis while obtaining active nuclei. Additional work has led to a reproducible and specific assay when compared to negative controls (use of NIH3T3 cells that do not express HER2/neu). Assessment of DNA binding agents for their ability to inhibition gene expression in this more sensitive assay that eludes the long mRNA half-life problem is currently underway.

Appendix:


1) Key Research Accomplishments:

- Task 1, months 6-12: half complete
 - ◆ Half of drugs in study are completed for prevention of complex
 - ◆ Future – all drugs in study for disruption of pre-formed complex
 - ◆ Results:
 - When comparing different modes of binding among the DNA binding agents tested thus far there does not seem to be a mode that is more potent for inhibition of transcription factor/DNA complex prevention. The exception being the polyamides. There may be a difference in potency of agents with different binding motifs when looking at the disruption of pre-formed complex.
 - When comparing sequence preference there does not appear to be a difference in potency when comparing AT rich binders to GC rich binders. However, the sequence specific agents inhibit complex formation at almost one order of magnitude greater than preference binding agents. Though Polyamide 22 is about 4X less potent than the parent compound Polyamide 2.
- Task 2, months 13-24: half complete
 - ◆ Half of drugs in study are completed for testing of inhibition of gene expression in cell-free transcription assay
 - ◆ Future – finish DNA binding agents using this assay
 - ◆ Results:
 - When comparing various modes of binding and binding preference among the DNA binding agents tested thus far there does not appear to be an order of magnitude of difference in potency.
 - For Polyamide 22 it appears to be about 4X less potent than the parent compound Polyamide 2.
- Task 3, months 6-36: addition of 2 assay – which I have developed for use in lab
 - ◆ Northern analysis
 - Optimized for pertinent use for our studies
 - Differences in mode of binding of agents tested:

Intercalators:	no effect
Minor groove binders:	varying effect
 - Differences in sequence or preference of binding of minor groove binders tested:

AT rich:	Hoechst's 33342	potent
	Distamycin	less potent
GC rich:	Chromomycin	most potent
 - Difference in agents for preference of targeted gene vs. general transcription inhibition of minor groove binders tested:

	<u>HER2 preference</u>	<u>General</u>
AT rich: Hoechst's 33342		X
Distamycin	X	
GC rich: Chromomycin	X	

- 
- ◆ Nuclear run-on
 - Optimized for pertinent use for our studies
 - Studies are currently underway
 - Results: none at this time
 - Addition of novel sequence specific DNA binding agent, polyamides, to consortium of drugs tested
 - ◆ Definitive proof that novel agents are cell permeable
 - Created/developed protocol for using fluorescence labeled agents in the lab
 - Developed understanding of equipment and programs necessary for detection and analysis

2.) Reportable Outcomes:

- We anticipate submission of a manuscript by the end of 1999.

3.) Copies of Reportable Outcomes:

- None at this time.



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

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26 Nov 02

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